LETTER TO JMG

Spastin mutations are frequent in sporadic spastic paraparesis and their spectrum is different from that observed in familial cases

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Background: *SPG4* encodes spastin, a member of the AAA protein family, and is the major gene responsible for autosomal dominant spastic paraplegia. It accounts for 10–40% of families with pure (or eventually complicated) hereditary spastic paraparesis (HSP).
Objective: To assess the frequency of *SPG4* mutation in patients with spastic paraplegia but without family histories.
Methods: 146 mostly European probands with progressive spastic paraplegia were studied (103 with pure spastic paraplegia and 43 with additional features). Major neurological causes of paraplegia were excluded. None had a family history of paraplegia. DNA was screened by DHPLC for mutations in the 17 coding exons of the *SPG4* gene. Sequence variants were characterised by direct sequencing. A panel of 600 control chromosomes was used to rule out polymorphisms.
Results: The overall rate of mutations was 12%; 19 different mutations were identified in 18 patients, 13 of which were novel. In one family, where both parents were examined and found to be normal, the mutation was transmitted by the asymptomatic mother, indicating reduced penetrance. The parents of other patients were not available for analysis but were reported to be normal. There was no evidence for de novo mutations. The mutations found in these apparently isolated patients were mostly of the missense type and tended to be associated with a less severe phenotype than previously described in patients with inherited mutations.
Conclusions : The unexpected presence of *SPG4* gene mutations in patients with sporadic spastic paraplegia suggests that gene testing should be done in individuals with pure or complicated spastic paraplegia without family histories.

Hereditary spastic paraplegias (HSP) are a genetically heterogeneous group of neurodegenerative disorders clinically characterised by progressive stiffness and weakness of the lower limbs. These symptoms are the result of axonal neurodegeneration of the cortico-spinal tract. Pure and complicated forms of HSP have been described, depending on whether spasticity occurs in the absence or the presence of other clinical features such as cerebellar ataxia, neuropathy, retinal degeneration, cognitive impairment, dementia, or epilepsy.

The clinical heterogeneity of HSP is partly explained by a large genetic heterogeneity. To date, at least 26 loci have been identified, associated with autosomal dominant, autosomal recessive, and X linked modes of inheritance. The most common form of autosomal dominant HSP (AD-HSP) is caused by mutations in the *SPG4* gene, encoding spastin, a member of the AAA protein family (AAA, ATPases associated with diverse cellular activities). More than 150 mutations have been identified all along the *SPG4* gene, including missense, nonsense, and splice site mutations, as well as frameshifts and larger deletions. *SPG4* has been shown to account for 15–40% of all autosomal dominant HSP families, depending on the population.[1–4] Emerging evidence suggests a role for spastin in microtubule dynamics,[5–9] but the mechanisms by which spastin abnormalities lead to axonal degeneration remain largely unknown.

Based on the observation that many mutations reduce the abundance of the normal full length transcript or functionally normal spastin protein, the pathogenic mechanism is likely to be haploinsufficiency—that is, loss of function rather than a dominant negative effect. HSP caused by *SPG4* mutations is generally described as a pure form of the disease—that is, as spastic paraparesis often associated with decreased vibration sense in the lower limbs and urinary problems. There is evidence that, at least in some cases, *SPG4* mutation carriers express a more complicated phenotype associated with cognitive impairment,[10–13] or in very few cases with cerebellar signs or epilepsy.[14–18] Age at onset is highly variable, ranging from early infancy up to the eighth decade.

In addition to age dependent penetrance, estimated at 85% by age 45, there are asymptomatic carriers who are still completely normal or have abnormal signs only when examined, even at 76 years of age.[1] To date, genetic testing for *SPG4* remains limited to patients with familial histories, consistent with autosomal dominant or at least dominant transmission. In contrast, the frequency of *SPG4* mutation carriers has not been systematically evaluated among isolated cases without family histories, and the question of de novo mutations has not been addressed. In the present study, we screened the *SPG4* gene in 146 isolated patients by denaturing high performance liquid chromatography (DHPLC), to determine the frequency of *SPG4* mutations among patients without family histories.

METHODS

Patients

We included 146 cases with pyramidal signs present predominantly in the lower limbs who were referred by neurologists after exclusion of neurological causes such as multiple sclerosis, other leucodystrophies, intramedullar tumours, primary lateral sclerosis, or adrenoleucodystrophy when appropriate. This allowed us to include probands presenting either pure (n = 103) or complicated (n = 43)

Abbreviations: AAA, ATPases associated with diverse cellular activities; DHPLC, denaturing high performance liquid chromatography; HSP, hereditary spastic paraparesis; SP, spastic paraparesis

Table 1 Primers, polymerase chain reaction and denaturing high performance liquid chromatography conditions

Exon	Forward/reverse primer	Annealing temperature	DHPLC temperature
1–1	GTTCCCGTCGGTCTGCGGGA/GAAGCGCTGGCAGAGCCAC	65	63.8°C/67.4°C
1–2	CCTTCCACCTGGGGCTCCTC/AGAAAAGGGACGCAGGTGTTGCC	65	64°C/66.8°C
2	GCATGATTTGCAATATTTAGTG/TAAATAGATCTGAAATCTGG	53	54°C
3	TTCTGTATAAAGACTGTGAC/CCACATTTTCAATCACTGATC	53	53.7°C
4	ATTTGTCATTTCACATGCAC/CATTATCAGGTTAAGTAAGAC	53	54°C
5	CCTTGGTTTTACAAATGTTTGC/ACTTAAGCAGGAATAGTATC	53	57°C/58°C
6	GAAAAGTGTAAATGTTAGGTTG/CACTCCTGACATGTTTTATAAG	53	52°C
7	GCTTCATCTTGTAATAACTG/TACTACTACTATGGATTCAG	53	54.5°C
8	TGTTTGGGAAGATGCTACTG/CAAGGACAAGATAAAGTTTC	53	54.2°C/56.6°C
9	TAATTTAATATTTGCTCTTG/AATACGACAATATTGGAAAC	53	52°C
10	ATTAAATTCCTGTGTGCTAG/TCTTTCCTATTTGGAGAGGG	53	54.5°C
11	CTCAGATGACTCACATAGC/AACCTTGGCCATTTAATAGG	53	54.5°C
12	TTCCTATTAAATGGCCAAGG/ATGTAAGATGGACACATGAG	53	53.5°C
13	CTTTTCCTGTCATTTGCTGTTT/GATGGTAGTTCTTGTTTCTGCTC	57	54.5°C
14	TAACAGCACAAGACCCTGTC/CTCATTTCATACTTAAGATTAC	53	53°C
15	ATAATCCCAGCTACCTGAG/TTGGACTTCTTAAACTTC	57	58.6°C
16	CCTTCAACAATTTCAACTGC/CACATTCATATATGTATGTC	53	51.5°C
17	TACTTTAATCCATCATTTCG/ATGACGTTCATTGAAGATC	53	55.5°C/57°C

DHPLC, denaturing high performance liquid chromatography.

spastic paraplegia. The complicated forms included cerebellar signs or atrophy on cerebral imaging (n = 15), signs of peripheral neuropathy on electromyography and conduction velocity studies (n = 14), cognitive impairment (n = 3) and mental retardation (n = 5) on neuropsychological testing, ophthalmoplegia (n = 3), or tremor n = (3).

We determined the absence of family history of the disease for each patient by systematic interview of the probands on their first and second degree relatives. In 18 cases, there was doubt as to a possible gait disturbance in first degree (n = 12), second degree, or even more distant relatives (n = 6), but information from the proband was insufficient to conclude that there was a familial spastic paraparesis. As the relatives were deceased or not available for examination, these cases were classified clinically as sporadic. Both parents were examined and were found normal in 11 families. In six families, only one parent was available for clinical examination, and in 28 cases, at least one parent had died before the age of 50. The patients were mostly of European origin (France (122), Italy (4), Portugal (3), Spain (2), Greece (1), Poland (1)), but 11 patients were from other geographical regions (North Africa (4), elsewhere in Africa (2), West Indies (1), Madagascar (1), Turkey (2), Asia (1)). Two had been adopted. In addition, 300 European and 100 North African controls (mostly healthy spouses of patients with other neurological diseases) were included to test new variants of the spastin gene.

Informed written consent was obtained from each individual before blood sampling. This study was approved by the ethics committee Paris-Necker (CCPPRB No 03-12-07, 2/10/2004).

DHPLC screening

To evaluate the frequency of *SPG4* patients in isolated cases with spastic paraplegia, we analysed the 17 coding exons of the *SPG4* gene by denaturing high performance liquid chromatography (DHPLC). Experimental conditions for DHPLC mutational analysis of the coding region of the spastin gene were first set up with DNA from 30 patients with well characterised mutations in each of the *SPG4* exons. All mutations were detected by DHPLC at the temperatures selected for the analysis.

The whole coding region of the *SPG4* gene was amplified by polymerase chain reaction (PCR), using 18 primer pairs. Formation of hetero-duplexes was enabled before DHPLC analysis by denaturation (five minutes at 95°C) followed by gradually cooling to 25°C. DHPLC analysis was carried out at a flow rate of 1.5 ml/min for a time period of 2.5 minutes on a WAVE DNA fragment analysis system HSM 3500HT (Transgenomic, Omaha, Nebraska, USA). The temperature of the column was set to the exon specific melting temperatures for successful resolution of hetero-duplexes. Wild-type samples were always used as negative controls, to ensure that a normal homo-duplex profile was reproducibly obtained with regard to retention time and peak profile. Chromatograms from each patient were overlaid with one from a normal individual. Samples with extra peaks or with a difference in peak appearance were scored as positive. Primers and DHPLC conditions are listed in table 1.

Sequence analysis

Samples showing abnormal elution profiles were re-amplified from genomic DNA. Both forward and reverse sequence reactions were done using the Big Dye Terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, California, USA). The sequence products were analysed on an ABI 3730 automated sequencer (PE Applied Biosystems).

Statistical tests

Frequencies were compared with the χ^2 test or Fisher's exact test when appropriate. Quantitative variables were compared by analysis of variance (ANOVA). Statistical analysis was done using SPSS software.

RESULTS

We identified 19 *SPG4* mutations at the heterozygous state in 18 of the 146 sporadic cases with spastic paraplegia, for a frequency as high as 12% (table 2).

The frequency of *SPG4* mutations was higher (16/103, 15.5%) in patients with a pure phenotype than in those with a complicated form of the disease (2/43, 4.6%) (p = 0.1). Both the latter had cognitive impairment (table 3B). Ages at onset ranged from childhood up to 70 years, with a mean (SD) age of 28.6 (17.9) years (excluding two patients with onset in childhood at an undetermined age). The overall severity was moderate after a mean duration of the disease of 17.4 (11.9) years (table 3A). One patient used a wheelchair after a disease duration of about 60 years. Interestingly, she had a complicated form of HSP (table 3B).

We identified 14 missense mutations (74%), three splice site mutations (16%), one nonsense mutation (5%), and one

Table 2 *SPG4* mutations in patients with sporadic spastic paraplegia

FN*	Origin	Mutation type	Exon/intron	Nucleotide change	Consequence	Polymorphism
134	France	Missense	Ex 1	c.127G→C	Glu43Gln	
521	North Africa	Splice site	Intr 1	c.415+1 g→a	Presumed missplicing	
175	Italy	Splice site	Intr 1	c.415+1 g→t	Presumed missplicing	
1619	France	Missense	Ex 5	c.712 C→A	p.Pro238Thr	
306	Portugal	Missense	Ex 8	c.1108 G→A	p.Gly370Arg	
278	France	Missense	Ex 8	c.1153 G→T	p.Gly385Trp	
91	France	Splice site	Intr 8	c.1173+1 g→a	Presumed missplicing	
113	France	Missense	Ex 9	c.1216 A→G	p.Ile406Val	
		Missense	Ex 17	c.1735 A→C	p.Asn579His	
004	France	Missense	Ex 10	c.1270 A→G	p.Arg424Gly	
048	France	Missense	Ex 11	c.1332 T→G	p.Asp444Glu	c.879 G→A/p.Pro293Pro
1610	France	Frameshift	Ex 11	c.1348_1352 del5	p.Glu452GlyfsX456	
213	France	Missense	Ex 13	c.1378 C→T	p.Arg460Cys	
207	France	Missense	Ex 13	c.1382 T→C	p.Leu461Pro	
154	Turkey	Missense	Ex 13	c.1495 C→T	p.Arg499Cys	
539	France	Missense	Ex 13	c.1495 C→T	p.Arg499Cys	
453	France	Missense	Ex 13	c.1496 G→A	p.Arg499His	c.131 C→T/p.Ser44Leu
124	France	Missense	Ex 13	c.1507 C→T	p.Arg503Trp	
008	France	Nonsense	Ex 17	c.1741 C→T	p.Arg581X	

*Family number.

frameshift (5%) (table 2). Four of the missense mutations (p.Gly370Arg, p.Arg424Gly, p.Arg460Cys, and p.Arg499Cys) have been described previously in AD-HSP families in which they were shown to segregate with the disease.[1 6] All other mutations were novel. In order to exclude the possibility that these novel heterozygous variations were in fact rare polymorphisms, we screened a healthy white population of 600 chromosomes. None of the variations was detected in the controls or previously described in patients, suggesting that they are likely to be involved in the HSP phenotype. The mutation c.415+1 g→a, identified in a patient from North Africa, was also absent from 200 chromosomes of North African controls. The c.1348_1352del5 and p.Arg581X mutations are both presumed to lead to a premature stop codon and a truncated protein, compatible with a deleterious effect and loss of spastin function. The p.Pro238Thr, p.Gly385Trp, p.Ile406Val, p.Asp444Glu, p.Leu461Pro, p.Arg499His, p.Arg503Trp, and p.Asn579His mutations modify highly conserved amino acids in mouse, rat, and pig spastin orthologues. While p.Pro238Thr is in a domain conserved only in mammals, all other mutations modified amino acids that are also conserved in fish and arthropods, suggesting that they are essential for spastin function.

Interestingly, one patient had two different missense mutations (p.Ile406Val and p.Asn579His) that were not found in any other patient. Unfortunately, only DNA of the patient was available, so we were unable to determine whether these mutations were inherited in *cis* (on the same allele) or *trans* (each on a different allele). If the mutations were inherited in *trans*, the association of these mutations could be responsible for the disease and therefore for its isolated nature in this patient. Another possibility is that only one of these mutations is causative whereas the other is only a very rare polymorphism.

In addition to the 19 *SPG4* mutations, we found one synonymous base change (c.390C→A/p.Ala130Ala, patient 39) and four different intronic variations close to an exon-intron boundary in six patients (c.1494-3dupt, c.1728+32c→g, c.1729-20t→a, and c.1496+18g→t in two patients; table 4). They were not detected on 600 control chromosomes, but until it is proven that they have an effect on the *SPG4* expression or splicing we consider them to be non-pathogenic.

In addition to the 25 variants not found in controls, we identified heterozygous variants in seven patients which were also found in the controls (tables 2 and 4). In four patients, a previously described synonymous polymorphism in exon 6 (c.879G→A/p.Pro293Pro) was detected. In the remaining three, a non-synonymous polymorphism, c.131C→T/p.Ser44Leu, was found in exon 1. The frequencies of c.879G→A/p.Pro293Pro and c.131C→T/p.Ser44Leu were similar in the HSP patients (A879/Pro293: 3/146 = 2%; T131/Leu44: 3/146 = 2%) and in the control population (A879/Pro293: 10/300 = 3.3%; T131/Leu44: 8/300 = 2.7%). The p.Ser44Leu allele was first described as a causal mutation at the homozygous state,[6] but was recently shown to be a polymorphism present in the general population which might act as a modifier of the HSP phenotype, especially the age of onset, when associated with a mutation.[19] Two of the carriers of these polymorphisms also had causative mutations: patient 048 had the c.879G→A/p.Pro293Pro polymorphism associated with the p.Asp444Glu mutation, and patient 453 had the non-synonymous p.Ser44Leu polymorphism combined with a p.Arg499His mutation. In the latter, the age at onset was very early (childhood) and the disease severe after 40 years of evolution.

We then examined whether our patients had apparently isolated disease because of insufficient genetic or clinical data (for example, the early death of parents, adoption, or small families). Eighteen of the probands reported a very questionable gait disturbance in another family member who was deceased or unavailable for examination. As information given by the proband was insufficient to formally conclude that the disease was familial, these patients were classified as sporadic (table 5). Five mutations were identified in these 18 patients (28%), three of which were second degree relatives or even more distantly related. This suggests that even weak evidence for a secondary case with HSP should be taken into account for molecular testing. Five mutation carriers (15%) were found in the 34 patients in whom one parent had died before the age of 50 (n = 28) or was unavailable for clinical examination (n = 6). More surprisingly, we identified one mutation (9%) among the 11 patients whose parents were both normal on clinical examination (table 5). Analysis of parental status in this family (008) showed that the p.Arg581X mutation was transmitted by the mother who was asymptomatic at age 67, excluding the possibility of a de novo event.

We were intrigued by the large proportion of missense mutations in our series compared with previously studied European populations with AD-HSP, in which missense mutations represented approximately 30% of all *SPG4* mutations and were generally clustered in the AAA cassette.[1] In our study, 74% (14/19) of the mutations were missense,

Table 3 Clinical and familial characteristics of sporadic spastin mutation carriers with (A) pure and (B) complicated hereditary spastic paraparesis

FN/sex	Parents and relatives (ages at death or examination (years))	Onset/duration (years)	Disability*	Spasticity/weakness	Increased reflexes/extensor plantar response	Other
(A) Sporadic spastin mutation carriers with pure HSP						
4/F	Parents NE; both deceased (62, 59)	34/13	4	Mild/none	LL increased; UL N/yes	
8/M	Parents E, N; both deceased (72, 77)	35/14	3	Severe/moderate	LL increased; UL N/yes	Slight scoliosis
48/M	Father deceased (52); mother well NE; mat aunt doubtful gait difficulties since age 15	12/36	3	Moderate/none	LL, UL increased/yes	Instability
91/M	Parents NE	20/14	3	Severe/none	LL increased; UL N/yes	
113/M	Parents NE	35/9	3	Severe/none	UL, LL increased/yes	
134/M	Parents NE deceased (73, 82); father had doubtful gait difficulties	41/8	3	Moderate/mild	UL, LL increased/yes	
154/M	Parents NE	3/12	3	Mild/none	LL increased; UL N/yes	
175/M	Father (60) E, N; mother NE (73)	13/20	4	Severe/none	UL, LL increased/yes	
207/F	Father unknown; mother NE (62)	10/21	2	Moderate/mild	UL, LL increased/yes	
213/M	Parents NE (63, 63)	41/5	1	Mild/none	LL increased; UL N/yes	
278/M	Mother E, N (53); father NE (52)	6/20	2	Severe/none	LL increased; UL N/yes	
306/F	Father deceased (67); mother E, N (77); mat GF had doubtful gait difficulties	20/25	2	Moderate/none	UL, LL increased/yes	
453/M	Parents deceased (63, 53); mat GM doubtful gait difficulties	Childhood/>40	3	Severe/moderate	Not determined	Pes cavus
521/M	Parents NE	43/7	2	Severe/none	LL increased; UL N/yes	
1610/M	Father NE, deceased (67); mother E, N (63)	28/8	2	Moderate/none	UL, LL increased/yes	
1619/M	Parents NE; both deceased (49, 67)	46/1	2	Moderate/none	LL increased/yes	
(B) Sporadic spastin mutation carriers with complicated HSP						
539/F	Parents NE (85, 87); father had doubtful gait difficulties	Childhood/60	6	Severe/severe	LL increased; UL N/yes	Pes cavus, scoliosis, acute onset of confusion, frontal behaviour at age 62
124/M	Parents both deceased (39/57)	70/9 (died 80)	3	Severe/moderate	LL increased; UL decreased/yes	Wasting LL, abolished vibration sense, severe cognitive impairment (MMS 8/30), focal epilepsy, axonal neuropathy, abnormal EEG, hypogonadism

*Disability was scored as: 1, no functional handicap but signs on examination; 2, mild, able to run, walking unlimited; 3, moderate, unable to run, limited walking without aid; 4, severe, walks with one cane; 5, walks with two canes; 6, unable to walk, needs a wheelchair.
E, examined; F, female; FN, family number; GF, grandfather; GM, grandmother; HSP, hereditary spastic paraparesis; LL, lower limb; M, male; mat, maternal; N, normal; NE, not examined; pat, paternal; UL, upper limb.

Table 4 *SPG4* variations with unknown effect and polymorphisms in patients with sporadic spastic paraplegia

FN	Origin	Type	Exon/intron	Nucleotide change	Consequence	Polymorphism
39	France	Synonymous	Ex 1	c.390 C→A	p.Ala130Ala	
82	France	Intronic	Intr 16	c.1728+32 c→g	Unknown	
330	France	Intronic	Intr 16	c.1729-20 t→a	Unknown	
637	France	Intronic	Intr 12	c.1493+18 g→t	Unknown	–
308	France	Intronic	Intr 12	c.1493+18 g→t	Unknown	
141	France	Intronic	Intr 12	c.1494-3 dupt	Unknown	
78	France	–	–		–	c.131 C→T/p.Ser44Leu
320	France	–	–		–	c.131 C→T/p.Ser44Leu
257	France	–	–		–	c.879 G→A/p.Pro293Pro
649	France	–	–		–	c.879 G→A/p.Pro293Pro
1613	France	–	–		–	c.879 G→A/p.Pro293Pro

FN: family number.

two of which (p.Glu43Asp and p.Pro238Thr) were located outside the AAA cassette. In order to compare the frequencies of missense mutations versus truncating mutations, we pooled the published data and our own unpublished data. Missense mutations were more frequent among sporadic cases (72%, 13/18) than in familial HSP (31%, 49/160), and this difference was statistically significant ($p = 0.001$). In contrast, truncating mutations—found throughout the coding region of the gene—represent approximately 50% of the mutations in AD-HSP,[1] but were found in only two (11%) of our cases (c.1348_1352del5 and p.Arg581X). Furthermore, with the exception of the c.1348_1352del5 frameshift, all the non-missense mutations identified in our isolated probands were surprisingly uncommon. Two of the splice site mutations we identified are novel, and both affect the donor site next to exon 1. The p.Arg581X nonsense mutation was located at the C-terminus of the protein. As a milder phenotype or a lower penetrance could account for the isolated nature of our patients, we tested the hypothesis that mutations identified in this study might be associated with less severe disease. We compared 64 probands from *SPG4* AD-HSP families and 18 sporadic *SPG4* mutations carriers. Sporadic *SPG4* mutations carriers were less severely affected than *SPG4* familial HSP patients (40.6% of AD-HSP patients needed help with walking, compared with only 16.7% of sporadic patients; $p = 0.060$), although the mean (SD) age at onset in familial (28.6 (16) years) and sporadic cases (28.7 (17) years) was similar, as were the mean disease duration and the mean age at examination. Taken together, these results indicate that in isolated patients, *SPG4* mutations are associated with a milder spastic paraparesis phenotype (and potentially reduced penetrance), which is responsible for apparently sporadic HSP.

DISCUSSION

Molecular analysis of the *SPG4* gene in this series of 146 patients with spastic paraplegia but without family histories, and after exclusion of other major neurological causes, identified at least 18 mutations carriers. The proportion of *SPG4* mutation carriers who appear to be isolated cases is therefore 12%. Mutation screening was carried out using DHPLC, which has already been used by other groups successfully to screen mutations in the spastin gene.[20 21] Although this method is efficient and reliable, its sensitivity is not complete, and screening of the coding sequence only may miss mutations in intronic or regulatory sequences or large scale rearrangements.

Seventeen of the 25 different DNA variants identified were novel and none was found on 600 control chromosomes. The causative role of 18 of the 25 mutations has already been demonstrated ($n = 5$) or is highly probable because they are predicted to produce truncated spastin ($n = 2$) or spastin with a missense affecting a conserved amino acid ($n = 9$) or to alter splicing ($n = 2$). The remaining DNA changes were a synonymous base change and four intronic variants which could affect spastin mRNA splicing, but their consequences remain to be determined in reverse transcriptase PCR experiments.

Stratification according to available information on the family reveals that the frequency of spastin mutations carriers is greatest (5/18, 28%) when there is even a weak suspicion of a secondary case in the family. If the doubtful cases are excluded, the proportion is still 10% (13/128), suggesting that molecular diagnosis of *SPG4* is indicated for patients with sporadic spastic paraplegia, after exclusion of other frequent causes of spasticity. The observation of a higher frequency of spastin mutations in isolated cases with pure spastic paraplegia (16/103) than in those with complex disease (2/43) is a potentially useful indication for molecular diagnosis.

The apparent isolation of the patients we studied may have several explanations—for example, lack of clinical data for the family and censor effects because of the early death of a parent. In everyday clinical practice, the parents and distant relatives are rarely available for examination and sampling, especially when the clinical interview does not suggest a genetic basis for the disease. Furthermore, although de novo SPG4 mutations are possible, none has ever been identified. Mutations are therefore probably transmitted and the apparent sporadic nature of the patients results from reduced penetrance, as in family 8. Normal clinical examinations of

Table 5 Frequency of *SPG4* mutations according to the familial data

Category	Number of cases	Number of mutations	Proportion of SPG4 mutations (%)
Both parents without clinical signs at examination	11	1	9.1
One parent unavailable for examination (n = 6) or one parent died before age 50 (n = 28)	34	5	14.7
Neither parent available for examination	83	7	8.5
Putative SP in another family member	18	5	27.7
Total	146	18	12.3

SP, spastic paraparesis.

both parents is therefore insufficient to exclude the possibility of an *SPG4* mutation.

Our results indicate that missense mutations are more common in isolated than in familial cases and are associated with milder disease. Genotype–phenotype correlations have not been very informative so far[1]; however, penetrance was not analysed according to the nature and the location of the mutations in the *SPG4* gene and some of the mutations in sporadic cases might have a lower penetrance than mutations identified in families. Furthermore, in most studies, the theoretical mutation type was used for correlations studies, which is not very informative as presumed ''missense'' mutations may differ in their effects at the mRNA and protein level. The selection of apparently isolated patients in this study could thus have facilitated the detection of missense mutations associated with reduced penetrance and disease severity.

Our study supports the existence of negative severity modifiers, such as the p.Ser44Leu allele, which is also present in the control population. Although p.Ser44Leu is not a susceptibility factor for spastic paraparesis because its frequency is similar in HSP patients and controls, it could contribute, in association with an SPG4 mutation, to an earlier age at onset.[19] Four different families with a mutation associated with the p.Ser44Leu allele have been described in the literature on SPG4, and p.Ser44Leu in the homozygous state was reported to cause spastic paraparesis.[6 19 22] We found one patient (453) with this polymorphism and the p.Arg499His mutation who had an early age at onset as well as severe spasticity. We also identified one patient (113) with two different missense mutations (p.Ile406Val and p.Asn579His), who had severe spasticity. These two patients, of 146, suggest that the combination of the two variations could cause a form of the disease that is more severe than in other family members. This also raises the possibility that other intronic 5'UTR or regulatory sequence variations that were not detected by DHPLC might affect the expression of *SPG4* and modify the clinical phenotype. Other genetic, epigenetic, or environmental factors could also affect disease expression in sporadic *SPG4* mutation carriers. Dominant genes, such as *SPG3A*, might be involved as well, although no sporadic cases with this mutation have been reported.

These results have implications for genetic counselling, as the risk for offspring of sporadic patients with *SPG4* mutations is difficult to evaluate. In a few cases it is difficult to determine completely the pathological nature of the variants identified, and the interpretation of the molecular data has to be handled with care in clinical practice. In most cases, the mutation is inherited in a dominant manner, but is associated with reduced penetrance. However, the factors determining the clinical expression and severity of the disease are not yet known. Nevertheless, testing for *SPG4* mutations in patients with pure spastic paraplegia, even in the absence of a positive family history, would not only alert offspring of sporadic patients to the potential risk of HSP but would also help identify these factors.

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